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Stereospecificity of inositol hexakisphosphate dephosphorylation by *Paramecium* phytase

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InsP₆ is an abundant compound in many micro-organisms, plants and animal cells. Its function and route of synthesis are still largely unknown. Degradation of InsP₆ is mediated by phytase, which in most organisms dephosphorylates InsP₆ in a relatively non-specific way. In the micro-organism *Paramecium*, however, the enzyme has been shown to dephosphorylate InsP₆ to InsP₂ in a specific order, but its stereospecificity has not been established, i.e. the phosphates are removed in the sequence 6/5/4/3 or 6/5/4/1 or 4/5/6/1 or 4/5/6/3 [Freund, Mayr, Tietz and Schultz (1992) *Eur. J. Biochem.* 207, 359–367]. We have

isolated the InsP₄ intermediate and identified its absolute configuration as D-Ins(1,2,3,4)P₄. Furthermore, degradation of [3,5-³²P]InsP₆ yielded a ³²P-labelled InsP₂ isomer, D-Ins(2,3)P₂. These data demonstrate that *Paramecium* phytase removes the phosphates of InsP₆ in the sequence 6/5/4/1. Knowing the stereochemical course of the enzyme, it can be used to elucidate the route of InsP₆ synthesis, as it allows us to determine the specific radioactivity at individual positions of the molecule after pulse-labelling cells with [³²P]P_i *in vivo* or [γ-³²P]ATP *in vitro*.

INTRODUCTION

The pivotal role of Ins(1,4,5)P₃ in signal transduction is well established [1]. This compound is metabolized by several kinases and phosphatases to a large array of inositol-containing compounds [2]. In most organisms these compounds attain concentrations in the low micromolar range [3–5]. Some compounds, however, reach very high concentrations, notably InsP₆. In plants the concentration of InsP₆ is several millimolar [6,7]. In micro-organisms such as *Dictyostelium* [5,8] and *Paramecium* [9] the concentration of InsP₆ approaches 0.6 mM, whereas in animal cells its concentration is less but still substantial [10]. Despite the abundance and potential connection with the universal signalling molecule Ins(1,4,5)P₃, not much is known about its function and metabolism.

Investigations of the routes of InsP₆ synthesis would be helped by the availability of enzymes that dephosphorylate InsP₆ in a position-specific order. This would allow us to follow the kinetics of incorporation and release of radioactive phosphate at each position of the molecule after pulse-labelling cells with [³²P]P_i. Such experiments have been instrumental in establishing metabolic routes of many compounds *in vivo*. Unfortunately, most InsP₆-degrading enzymes are either not very specific or degrade InsP₆ only at one position [11–13]. An enzyme from *Paramecium* has been described [9] that degrades InsP₆ to D/L-Ins(1,2)P₂ via specific intermediates: D/L-Ins(1,2,3,4,5)P₅, D/L-Ins(1,2,3,4)P₄ and Ins(1,2,3)P₃. As the absolute configurations of these intermediates have not been determined, four potential dephosphorylation routes are possible: sequential dephosphorylation at 6/5/4/3, 6/5/4/1, 4/5/6/3 and 4/5/6/1.

In this paper we identify the configuration of the intermediate InsP₄ isomer as D-Ins(1,2,3,4)P₄, and show that the InsP₂ isomer has retained the phosphate at the 3-position. These observations establish the stereochemical course of the enzyme reactions as 6/5/4/1.

MATERIALS AND METHODS

Materials

Alkaline phosphatase (grade II; calf intestine), NAD⁺ and

hexokinase from yeast [(NH₄)₂SO₄ suspension] were from Boehringer-Mannheim. InsP₆, L-polyol dehydrogenase and Amberlite mixed-bed (MB3) were obtained from Sigma. The Zorbax SAX column and the Lichrosorb-NH2 column were purchased from Chrompack. Polycarbonate filters of 3 μm pore size were from Nuclepore. [¹⁴C]Glucose 6-phosphate (0.295 Ci/mmol), [2-³H]Ins(1,3,4)P₃ (2 Ci/mmol) and [γ-³²P]ATP (3000 Ci/mmol) were from Amersham. [2-³H]InsP₆ (23 Ci/mmol) was from NEN-Dupont.

Isolation of phytase from *Paramecium*

Phytase was isolated as described by Freund et al. [9], with minor modifications. *Paramecium* cells were homogenized at 4 °C with 25 strokes of a Potter homogenizer in 50 mM sodium acetate, pH 5.5, containing 5 mM EDTA, 5 mM EGTA, 2 mM benzamide, 0.2 mM PMSF and 0.02 % NaN₃. The homogenate was centrifuged for 30 min at 48 000 g and the pellet was resuspended to 20 mg of protein/ml in 50 mM Tris/HCl, pH 7.0. After 4 × 15 s sonication at 0 °C, the suspension was centrifuged for 60 min at 100 000 g. (NH₄)₂SO₄ (60 % satd.) was added to the supernatant, and the mixture was incubated for 1 h on ice and centrifuged for 1 h at 100 000 g. The resulting pellet was dissolved in 0.2 vol. and dialysed overnight at 4 °C against 1000 vol. of 50 mM Tris/HCl, pH 7.0.

Determination of the stereoselectivity of *Paramecium* phytase

[³H]InsP₆ (65 000 c.p.m.) was incubated in a total volume of 100 μl with 50 mM Tris/HCl, pH 7.0, and 20 μl of *Paramecium* phytase from the preparation described above. The reaction was terminated after 60 min by adding EDTA to a final concentration of 10 mM and subsequent boiling for 2 min. The [³H]InsP₄ isomer was isolated by HPLC using a Zorbax column eluted at 1 ml/min with a gradient consisting of water in pump A and 1.2 M ammonium phosphate, pH 3.7, in pump B with the following break points: 0 % B at 0 min, 5 % B at 1 min, 10 % B at 4 min, and 100 % B at 20 min. The salt from the pooled InsP₄ fractions was removed by dialysis against 10 mM Hepes, pH 7.1,

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(see below), after which the sample was lyophilized (yield 30%; 19 500 c.p.m.).

The absolute configuration of the InsP_4 isomer was determined after periodate oxidation, reduction and incubation with a stereospecific dehydrogenase [14–17]. Briefly, the purified InsP_4 (18 000 c.p.m.) was oxidized for 36 h with 0.5 ml of 0.1 M NaIO_4 , pH 2.0, at room temperature in the dark, followed by reduction in an open vessel for 12 h by addition of 0.5 ml of 0.5 M NaBH_4 . After dialysis and lyophilization, the yield of this step was 65% (11 500 c.p.m.). An aliquot of the sample (5000 c.p.m.) was dephosphorylated overnight at 37 °C with 0.25 mg/ml alkaline phosphatase in 0.1 M glycine buffer, pH 10.4, containing 1 mM ZnCl_2 and 1 mM MgCl_2 ; the reaction was terminated by the addition of 10 mM EDTA followed by boiling for 2 min. The sample was desalted using Amberlite mixed-bed resin (yield of this step 53%; 2600 c.p.m.).

The isolated ^3H -labelled polyol (400 c.p.m.) was incubated with the stereoselective L-polyol dehydrogenase for 2.5 h at room temperature in a reaction mixture containing 50 mM Tris/HCl, pH 8.3, 20 mM NAD^+ and 1 unit/ml L-polyol dehydrogenase. The reaction was terminated by boiling the sample for 2 min, followed by lyophilization. Analysis of the ^3H -labelled products was performed by reverse-phase HPLC using a Lichrosorb-NH2 column eluted with a gradient of acetonitrile and water. [^{14}C]Glucose, prepared from [^{14}C]glucose 6-phosphate with alkaline phosphatase, was included with each sample as an internal standard. $\text{Ins}(1,3,4)\text{P}_3$ was processed in an identical way as a positive control, as it yields the substrate L-altritol.

Dialysis of inositol polyphosphates

Owing to their strong polarity, compounds such as inositol polyphosphates are retained by dialysis membranes with a pore size 50-fold larger than their molecular mass [18]. After overnight dialysis against 1000 vol. of 10 mM Hepes, pH 7.1, the percentage of inositol, $\text{Ins}3\text{P}$, $\text{Ins}(1,4,5)\text{P}_3$ and InsP_6 retained in the dialysis bag was about 0, 15, 50 and 80% respectively. The difference between the rates of diffusion of polar inositol polyphosphates and uncharged molecules or small ions was used for effective desalting of the compounds of interest with optimal yield. This method is very efficient for compounds with three or more phosphates [18]. Thus dialysis of InsP_4 isomers was for 3 × 2 h against 1000 vol. of Hepes, pH 7.1, whereas dialysis of InsP_6 was for 3 × 6 h, both with a yield of approx. 75%.

Synthesis of $\text{Ins}[3,5\text{-}^{32}\text{P}]\text{P}_6$ using a *Dictyostelium* lysate and analysis with *Paramecium* phytase

Stephens and Irvine [19] described the dephosphorylation and ATP-mediated rephosphorylation of [^3H] InsP_6 at the 3- and 5-position in a lysate of *Dictyostelium* cells. $\text{Ins}[3,5\text{-}^{32}\text{P}]\text{P}_6$ was prepared using unlabelled InsP_6 and [$\gamma\text{-}^{32}\text{P}$]ATP. Wild-type AX3 cells were grown in modified HL5 medium containing 10 g/l D-glucose as described [20] and starved in 10 mM sodium/potassium phosphate buffer for 2 h at 10^7 cells/ml. Cells were harvested and washed once in 40 mM Hepes/0.5 mM EDTA, pH 6.5, and subsequently lysed at 4 °C by elution through a polycarbonate filter with a pore size of 3 μm [21].

The reaction mixture contained 1 μM InsP_6 , 3 nM [$\gamma\text{-}^{32}\text{P}$]ATP (1 μCi) and 50 μl of the *Dictyostelium* lysate in a total volume of 100 μl of 50 mM Hepes, pH 7.0. The reaction was terminated after 15 min by boiling for 2 min, and [^3H] InsP_6 (about 60 000 d.p.m.) was added. The mixture was applied to the Zorbax HPLC column eluted as described above. The fractions containing InsP_6 were pooled and dialysed overnight against

3 × 500 vol. of 10 mM Hepes, pH 7.1, to remove the ammonium phosphate.

This purified [^{32}P]/[^3H] InsP_6 mixture was dephosphorylated stepwise at the D/L 6-, 5-, 4- and 3-position using 20 μl of *Paramecium* phytase in 100 μl of 50 mM Tris/HCl, pH 7.0, containing about 5000 d.p.m. $\text{Ins}^{[32}\text{P}]\text{P}_6$ and 5000 d.p.m. [^3H] InsP_6 . The incubations were at room temperature for $t = 0$, 10 and 120 min and 16 h. The samples were analysed using the Zorbax HPLC column eluted as described above. Fractions of 20 s were collected and 4 ml of emulsifier 299 was added. Radioactivity was determined with a dual-label counting program and using a quench-correction curve.

RESULTS AND DISCUSSION

A detailed characterization of *Paramecium* phytase by Freund et al. [9] revealed that this enzyme degrades InsP_6 by stepwise dephosphorylation via D/L- $\text{Ins}(1,2,3,4,5)\text{P}_5$, D/L- $\text{Ins}(1,2,3,4)\text{P}_4$

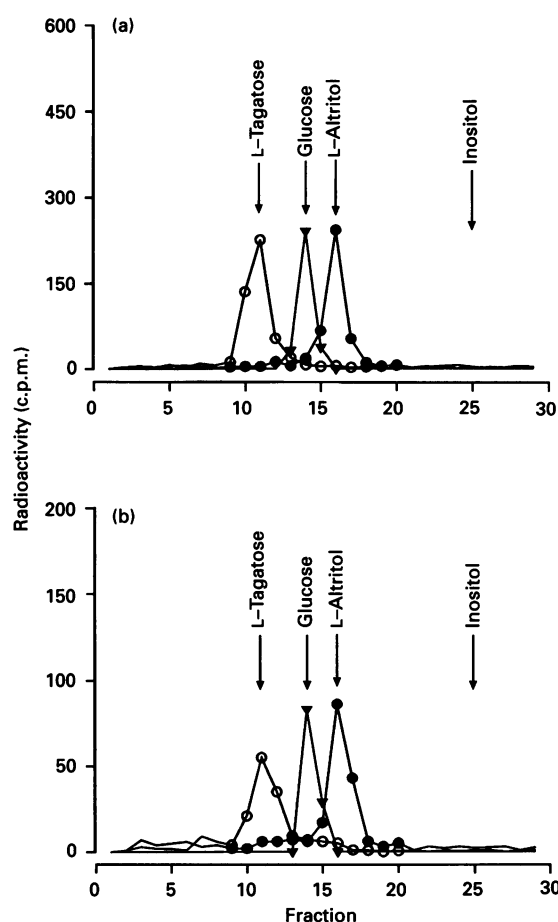


Figure 1 HPLC separation after oxidation of the polyol derived from authentic [^3H] $\text{Ins}(1,3,4)\text{P}_3$ (a) and [^3H] InsP_4 generated by degrading [^3H] InsP_6 with *Paramecium* phytase (b)

[^3H] InsP_6 was degraded with *Paramecium* phytase and the resulting [^3H] InsP_4 was isolated, oxidized, dephosphorylated and incubated with L-polyol dehydrogenase. The enzyme degrades only L-altritol derived from $\text{Ins}(1,2,3,4)\text{P}_4$ yielding L-tagatose, and not D-altritol derived from $\text{Ins}(1,2,3,6)\text{P}_4$. The elution patterns after incubation with boiled (●) or active (○) L-polyol dehydrogenase are presented; ▼, internal standard [^{14}C]glucose. Authentic [^3H] $\text{Ins}(1,3,4)\text{P}_3$ was treated in parallel as a positive control, as it yields L-altritol. The formation of L-tagatose from D/L- $\text{Ins}(1,2,3,4)\text{P}_4$ with no remaining altritol implies that the enzyme dephosphorylates InsP_6 exclusively via the sequential removal of the phosphates at the 6-, 5- and 4-positions.

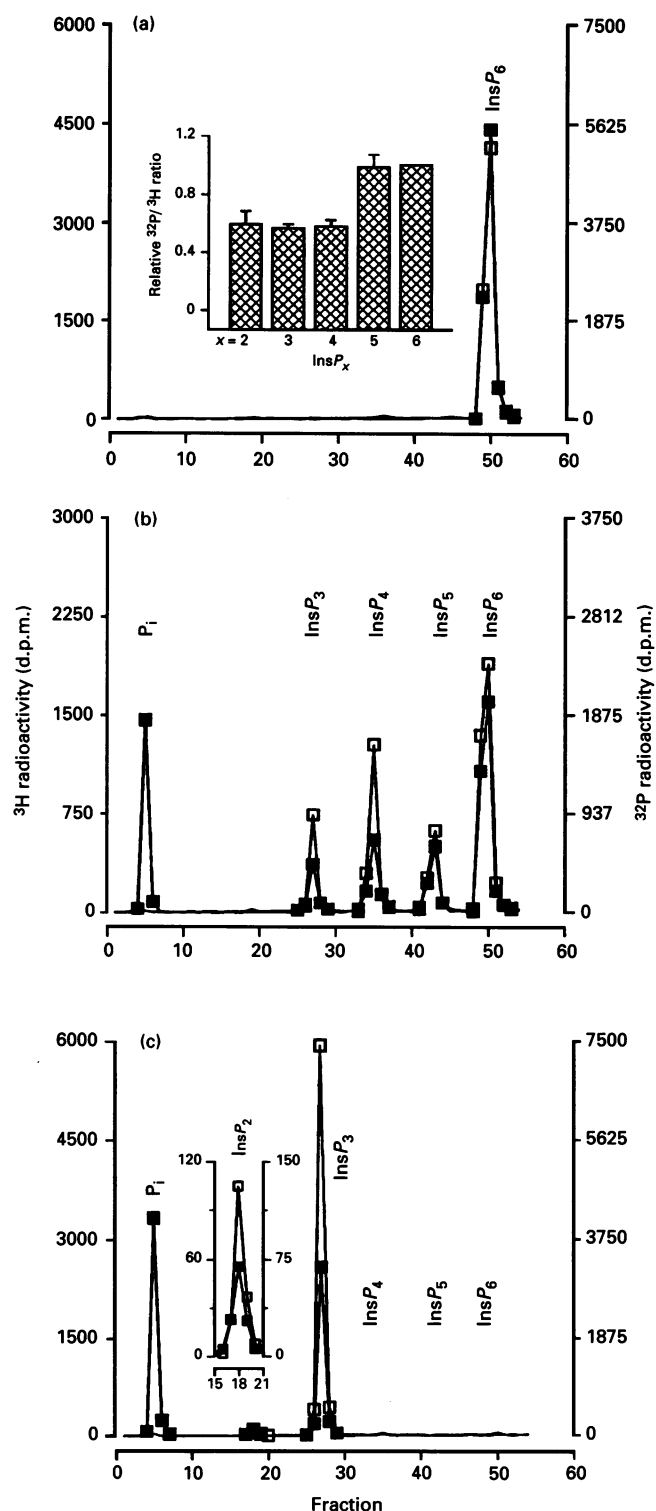


Figure 2 HPLC separation after degradation by *Paramecium* phytase of a mixture of [^3H]Ins P_6 and Ins[3,5- ^{32}P]P $_6$

Ins[3,5- ^{32}P]P $_6$ was prepared by phosphate exchange of InsP $_6$ with [γ - ^{32}P]ATP *in vitro* in a *Dictyostelium* lysate. The purified Ins[3,5- ^{32}P]P $_6$ was mixed with authentic [^3H]InsP $_6$ and incubated with *Paramecium* phytase for 0 min (a), 10 min (b) and 16 h (c) followed by separation of the reaction products by HPLC. The ordinates were scaled such that the [^3H]InsP $_6$ (□) and Ins[^{32}P]P $_6$ (■) peaks have approximately equal size (all panels have the same relative scaling). The inset in (a) reveals the $^{32}\text{P}/^3\text{H}$ ratio in the degradation products relative to that in InsP $_6$. The main figures are the means of two incubations of a typical experiment reproduced twice; the inset in (a) shows the means \pm S.D. of all experiments. The reduction of the ratio

and Ins(1,2,3)P $_3$ finally to D/L-Ins(1,2)P $_2$. Removal of the first three phosphates may occur via two routes: starting at the 6-position via the 5- to the 4-position (notation 6/5/4) or starting at the 4-position via the 5- to the 6-position (4/5/6). The next phosphate is removed from Ins(1,2,3)P $_3$ at either the 1- or 3-position. One may expect that the phytase continues dephosphorylation at the phosphate adjacent to the previously removed phosphates (i.e. 6/5/4/3 or 4/5/6/1). However, this assumption has not been formally validated. Thus, besides the adjacent dephosphorylations at positions 6/5/4/3 or 4/5/6/1, the two interrupted series 6/5/4/1 and 4/5/6/3 are also possible. Finally, it cannot be excluded that *Paramecium* phytase degrades InsP $_6$ via more than one of these routes. Two experiments were performed to discriminate between these four routes: determination of the absolute conformation of D/L-Ins(1,2,3,4)P $_4$, and analysis of the phosphorylation state of the 3-position in D/L-Ins(1,2)P $_2$.

Stereoselective assignment of inositol phosphates can be established by the procedure originally developed by Ballou and co-workers [14,15] and extended by Stephens et al. [16,17]. This procedure includes oxidation with periodate (cleavage between two vicinal hydroxy groups), followed by reduction and dephosphorylation. The polyol produced is then incubated with L-polyol dehydrogenase and the products are characterized by HPLC. By following this procedure, the InsP $_4$ isomer produced from [^3H]InsP $_6$ by *Paramecium* phytase was identified, and is either D-Ins(1,2,3,4)P $_4$ yielding L-altritol which is oxidized to L-tagatose, or D-Ins(1,2,3,6)P $_4$ [= L-Ins(1,2,3,4)P $_4$] yielding D-altritol which is not oxidized by L-polyol dehydrogenase. In these experiments authentic [^3H]Ins(1,3,4)P $_3$ was treated in parallel, as it yields L-altritol. This provided a positive control for the L-polyol dehydrogenase reaction and for the resolution of the HPLC system. The experiments reveal that the polyol derived from [^3H]Ins(1,3,4)P $_3$ (i.e. L-[^3H]altritol) was eluted after the internal standard [^{14}C]glucose (Figure 1a). On incubation of this L-[^3H]altritol with active L-polyol dehydrogenase, a ^3H -labelled product was detected that was eluted before [^{14}C]glucose (i.e. L-[^3H]tagatose). Thus the experiments with Ins(1,3,4)P $_3$ confirm the procedure and the separation potential of the reverse-phase HPLC column.

[^3H]InsP $_6$ was incubated with *Paramecium* phytase and the [^3H]InsP $_4$ isomer was isolated by HPLC. After oxidation and reduction, the ^3H -labelled polyol was eluted at the expected position of L/D-altritol (Figure 1b). On incubation of this polyol with active L-polyol dehydrogenase, a ^3H -labelled product was detected that was eluted before [^{14}C]glucose at the position of L-tagatose. Since L-tagatose must be derived from L-altritol, the InsP $_4$ isomer was D-Ins(1,2,3,4)P $_4$, and not D-Ins(1,2,3,6)P $_4$, which would have yielded the non-degradable D-altritol. The absence of ^3H -labelled altritol after oxidation with L-polyol dehydrogenase indicates that the *Paramecium* phytase dephosphorylated InsP $_6$ exclusively in one direction, starting from the phosphate at position 6 in the sequence 5 and 4.

The next dephosphorylation step was expected to occur at the 3-position, adjacent to the last dephosphorylation at the 4-position [9]. However, it cannot be excluded that the 1-phosphate is removed. This was investigated by analysing the presence of the 3-phosphate in the InsP $_2$ product. It has been demonstrated that InsP $_6$ is rapidly dephosphorylated and rephosphorylated at the 3- and 5-positions in a *Dictyostelium* lysate [19]. Thus

at a specific dephosphorylation step indicates the fraction of ^{32}P radioactivity at that phosphate position. The absence of radioactive phosphate release during the dephosphorylation of [^{32}P]Ins(1,2,3)P $_3$ implies that exclusively Ins(2,3)P $_2$ is formed.

incubation of a *Dictyostelium* lysate with InsP_6 and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ yields $\text{Ins}[3,5\text{-}^{32}\text{P}]\text{P}_6$. This compound was isolated by HPLC, desalted by dialysis, and mixed with authentic $[\text{H}]\text{InsP}_6$. The $^{32}\text{P}/^3\text{H}$ ratio of InsP_6 was 1.176 (Figure 2a). After a short incubation period with *Paramecium* phytase, mainly $\text{Ins}(1,2,3,4,5)\text{P}_5$ and $\text{Ins}(1,2,3,4)\text{P}_4$ were formed (Figure 2b). The $^{32}\text{P}/^3\text{H}$ ratio of $\text{Ins}(1,2,3,4,5)\text{P}_5$ was 1.162, which is 0.988 ± 0.086 relative to the ratio in InsP_6 , indicating the absence of ^{32}P radioactivity at the 6-position. In contrast, the $^{32}\text{P}/^3\text{H}$ ratio of $\text{Ins}(1,2,3,4)\text{P}_4$ was only 0.579 ± 0.045 relative to the ratio in InsP_6 , revealing that $42 \pm 5\%$ of the ^{32}P radioactivity in InsP_6 was located at the 5-position. On longer incubation of InsP_6 with phytase, mainly $\text{Ins}(1,2,3)\text{P}_3$ and small amounts of D/L- $\text{Ins}(1,2)\text{P}_2$ were produced (Figure 2c). The $^{32}\text{P}/^3\text{H}$ ratio of $\text{Ins}(1,2,3)\text{P}_3$ was 0.566 ± 0.031 relative to the ratio in InsP_6 , which is essentially identical with the ratio of $\text{Ins}(1,2,3,4)\text{P}_4$, indicating the absence of ^{32}P radioactivity at the 4-position of InsP_6 . Finally, at the position of InsP_2 a compound was eluted containing both ^{32}P and ^3H radioactivity. The $^{32}\text{P}/^3\text{H}$ ratio was 0.590 ± 0.095 relative to the ratio of InsP_6 , which is not significantly different from the ratio of $\text{Ins}(1,2,3)\text{P}_3$ (0.566 ± 0.031). This demonstrates that all ^{32}P at the 3-position of InsP_6 is retained in D/L- $\text{Ins}(1,2)\text{P}_2$, thereby identifying this compound as D- $\text{Ins}(2,3)\text{P}_2$. The observation that no significant ^{32}P radioactivity is released on degradation of $\text{Ins}(1,2,3)\text{P}_3$ indicates that at least 85% of dephosphorylation of this compound occurs at the 3-position and at most 15% at the 1-position (calculated at $P < 0.05$).

In summary, the combined data indicate that InsP_6 is dephosphorylated by *Paramecium* phytase in a stereospecific way by sequential removal of phosphates at the 6, 5, 4 and 1 position. Unfortunately, the *Paramecium* phytase does not effectively degrade InsP_6 further than $\text{Ins}(2,3)\text{P}_2$, so discrimination between labelling at the 2- and 3-positions is not possible. It should also be mentioned that degradation of $\text{Ins}(1,2,3)\text{P}_3$ to $\text{Ins}(2,3)\text{P}_2$ is very slow, which makes it difficult to obtain reliable data on the specific radioactivity of phosphate at the 1-position.

A knowledge of the absolute stereochemical specificity of the *Paramecium* phytase reaction allows us to use this enzyme to produce specific inositol phosphate isomers, notably $\text{Ins}(1,2,3,4,5)\text{P}_5$, $\text{Ins}(1,2,3,4)\text{P}_4$ and $\text{Ins}(1,2,3)\text{P}_3$. Moreover, the enzyme could be instrumental in assigning the sequence of phosphate incorporation at the different positions of InsP_6 *in vivo*. Towards this application of the enzyme, $[\text{H}]\text{InsP}_6$ is isolated at different times after labelling of cells *in vivo* with $[\text{H}]\text{P}_i$ in a pulse-chase protocol. The isolated $\text{Ins}[\text{H}]\text{P}_6$ is mixed with authentic $[\text{H}]\text{InsP}_6$ and incubated with *Paramecium* phytase. The reduction in the $^{32}\text{P}/^3\text{H}$ ratio of the degradation products relative to that of InsP_6 (Figure 2) provides direct information on the fraction of ^{32}P label present at each position, except positions

2 and 3. In pulse-chase experiments, positions that are phosphorylated *in vivo* at the end of the InsP_6 -synthetic pathway are labelled relatively fast (i.e. are relatively 'hot'), whereas relatively 'cold' phosphates have been added early in the pathway of InsP_6 formation. This method is used in the following paper [22] to discriminate between three potential routes of InsP_6 formation in *Dictyostelium* cells that have been observed to occur *in vitro*: (i) futile dephosphorylation/phosphorylation reactions at positions 3 and 5 [19]; (ii) sequential phosphorylation of inositol at positions 3, 6, 4, 1, 5 and 2 [19]; (iii) sequential phosphorylation of $\text{Ins}(1,4,5)\text{P}_3$ at positions 3, 6 and 2 [22]. We observed that $\text{Ins}[\text{H}]\text{P}_6$ isolated after a very brief labelling of cells with $[\text{H}]\text{P}_i$ has a higher specific radioactivity at the 6-position than at positions 4 or 5, demonstrating that the third route is preferred *in vivo* [22].

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